

ANTIMICROBIAL TEST LABORATORIES

Study ID: GLP1290

Client: Sterilex Corporation

Protocol Number: P1331

PROTOCOL

ANTIMICROBIAL TEST LABORATORIES

Protocol for GLP AOAC Use-Dilution Method
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Test Microorganism(s)
Listeria monocytogenes ATCC 15313

Product/Identity
Test Substance: Sterile Ultra Disinfectant Cleaner Solution 1
Lots: AM1-258, AM1-24A

Test Substance: Sterile Ultra Activator Solution
Lots: AM1-129A, AM1-129B

Data Requirement
US EPA 40 CFR Part 158
U.S. EPA OCS-P 810 2200

Study Sponsor
Edward Fu
Sterilex Corporation
111 Lake Front Drive
Hunt Valley, MD 21030

Performing Laboratory
Antimicrobial Test Laboratories
1304 W. Industrial Blvd.
Round Rock, Texas 78681

Protocol Number
P1331

Study Director:
Nicholas Garcia, B.S.

Date
19JUN2015

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I. Introduction

This document details the materials and procedure for evaluating the efficacy of liquid disinfectants using the AOAC Use-Dilution Method in accordance with Good Laboratory Practice Standards (GLPS) stipulated by 40 CFR 160. This document also explains the terms and conditions of testing.

II. Purpose

The purpose of this study is to document the efficacy of the test substance against the test system (microorganism) under the specified test parameters.

III. Justification for the Selection of Test System (Microorganism)

The United States Environmental Protection Agency (USEPA) requires specific anti-microbial claims made for disinfectants for use on hard surfaces and sold in the United States to be supported by relevant test systems (microorganisms) outlined in EPA Product Performance Test Guidelines, OCSP 810-2200, Disinfectants for Use on Hard Surfaces, Efficacy Data Recommendations and other related EPA guidance.

IV. Terms and Conditions

Studies by Antimicrobial Test Laboratories are conducted in accordance with general terms and conditions posted at www.AntimicrobialTestLaboratories.com/terms.htm

Prior to study initiation, Antimicrobial Test Laboratories must receive the approved and signed protocol, test substance and payment. Changes to the signed, approved protocol will require amendment and may incur additional fees. Cancellation of the study any time after the protocol has been signed will result in a cancellation fee of up to 100% of the total study cost, to be determined by laboratory management at its sole discretion.

Antimicrobial Test Laboratories may repeat studies, free of charge, in the event of unintended protocol non-conformance, if the non-conformance is determined by the Study Director to have affected the study outcome. If the characterization system specified for a study is not adequate, the study will be deemed "inconclusive" and the Study Sponsor will be responsible for the cost of the study. In addition, the Study Sponsor is responsible for the cost of all studies performed to confirm the outcome of a previous study and for ensuring that the study will meet the regulatory objectives.

The Study Sponsor must obtain written consent from Antimicrobial Test Laboratories to use or publish its protocols, study reports (or parts thereof), logo or employee names for marketing purposes.

Test substance characterization as to content, stability, etc., is the responsibility of the Study Sponsor. The test substance shall be characterized by the sponsor prior to the completion of this study.

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V. Test Substance Identification, Characterization, and Handling

All test substances used to substantiate antimicrobial efficacy claims will be manufactured or otherwise tested at the lower certified limit (LCL).

Test Substance Name — Sterile Ultra Disinfectant Cleaner Solution 1
Lot Number[s] — AM1-24A
Active Ingredient & Concentration — See attached Certificate of Analysis
Manufacture Date — 23FEB2015
Expiration Date - 23FEB2016

Test Substance Name — Sterile Ultra Disinfectant Cleaner Solution 1
Lot Number[s] - AM1-25B
Active Ingredient & Concentration — See attached Certificate of Analysis
Manufacture Date - 23FEB2015
Expiration Date - 23FEB2016

Test Substance Name — Sterile Ultra Activator Solution
Lot Number[s]: AM1-129B
Active Ingredient & Concentration — N/A
Manufacture Date — 03APR2015
Expiration Date — 03APR2016

Test Substance Name — Sterile Ultra Activator Solution
Lot Number[s]: AM1-129A
Active Ingredient & Concentration — N/A
Manufacture Date — 03APR2015
Expiration Date — 03APR2016

Special Handling Requirements — None

Test substance characterization as to content, stability, etc., 40 CFR, Part 160, and Sub part F (160.105) is the responsibility of the Study Sponsor. The test substance shall be characterized by the Sponsor prior to the completion of this study.

Test substances and devices are handled as follows.

- The test substance is stored at ambient [room] temperature under fluorescent lighting or in a cabinet.
- The test substance is shaken or otherwise mixed well immediately prior to use [if applicable].
- The test substance is handled safely in accordance with the chemical risks it may pose, stated in the MSDS or by the Study Sponsor during the course of pre-study communication.

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VI. Study Parameters, Incorporated by Reference

Number of Tests Comprising the Study — 2 (1 Test per Test Substance Lot per Test Microorganism)
Carrier Type — Stainless Steel Penicylindar
Number of Carriers per Test Substance — 10
Test Substance Form — Dilution Required (1:1-10), 1 part Sterile Ultra Disinfectant Cleaner Solution + 1 part Sterile Ultra Activator Solution + 10 parts Diluent
Test Substance Diluent — 400 ± 10 PPH AOAC Synthetic Hard Water Solution
Test Temperature — 20 ± 1°C
Contact Time — 9 Minutes ± 5 seconds
Organic Soil Load — 5 ± 0.1% (v/v) Fetal Bovine Serum (FBS)
Neutralization Broth — Dey/Engley (D/E) Broth supplemented with 0.1% catalase

Proposed Experimental Start Date: 19JUN2015
Proposed Experimental Termination Date: 19JUL2015

VII. Test System (Microorganism)

Listeria monocytogenes ATCC 15313

VIII. Materials

- Pure culture of the test system [microorganism]
- Sufficient quantity sterile 8 ± 1 mm od, 6 ± 1 mm id, 10 ± 1 mm length, type 304 stainless steel penicylinders free of visible flaws. For a 60 carrier test, at least 69 carriers are necessary per microorganism and lot of product tested (60 test carriers, 6 inoculum control carriers, 1 neutralization control carrier, and 2 viability control carriers). Extra carriers may be prepared for use in the study.
- Sufficient volume of reagent grade 1N NaOH solution.
- Sufficient quantity of clean, sterile 100×15 mm sterile Petri dishes.
- Sufficient quantity of sterile 9 cm Whistman #2 (or equivalent) filter paper rounds.
- Sufficient quantity of 20 x 150 mm test tubes containing 10 ml sterile AOAC Synthetic Broth or Nutrient Broth
- Sufficient quantity of sterile petri dishes
- Sufficient volume of sterile fetal bovine serum for addition of artificial "soil" load to microbial culture if applicable
- Sufficient clean, sterile 25×150 mm test tubes
- Sufficient sterile tubes containing sterile phosphate-buffered saline, for dilution of microbial suspensions prior to plating.
- Sufficient number and volume of sterile Petri dishes and sterile Tryptic Soy Agar or other appropriate growth agar for enumeration of diluted microbial suspensions
- Sufficient number of 25×150 mm test tubes containing 10 ml sterile subculture neutralization broth
- Two or more bent wire transfer hooks of a type that can be flame-sterilized quickly yet are strong enough to fully support the weight of a penicylinder during transfer from one tube to the next.

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- Bunsen burner, microbiological incubator, or micro-lorch as appropriate to ensure rapid and complete flame-sterilization of transfer hooks.
- Sufficient quantity of micropipettes and appropriately sized sterile micropipette tips.
- Automatic pipetor (Pipet-Aid or similar) and various sizes of sterile serological pipets.
- Thermometer (or submersion in an equilibrated test tube to indicate the temperature of the test substance during the test).
- Incubator capable of sustaining temperatures of $36 \pm 1^\circ\text{C}$.
- Forceps.
- Appropriate volume of 95% ethanol.
- Wire inoculating loop [4mm id].
- Sufficient number of test tube racks.
- Sonicator.
- Certified satellite clock.
- Certified digital timer.
- Water Bath capable of maintaining the appropriate test temperature.

D. Procedure

Preparation of AOAC synthetic hard water solution

- From each 1000 ml of sterile RO water (as measured by 1L volumetric flask), a volume equal to the total volume of AOAC hard water reagents added in the steps below is removed by serological pipette. For example, if 4 ml of solution "1" and 4 ml of solution "2" are to be added, then 8 ml of sterile water is removed.
- The concentration in PPM of hard water to be made is divided by 100. That is the volume, in ml, of AOAC hard water solutions "1" and "2" that will be needed to make 1000ml of hard water.
- Based on the calculation above, an appropriate volume of AOAC solution "1" is added to the sterile water, and mixed.
- The appropriate volume of solution "2" is then added and mixed.
- An appropriate volume of the synthetic hard water is removed and titrated. If necessary, the solution may be diluted with sterile water or augmented with equal parts solution "1" and "2" to achieve the study sponsor requested hard water level. In any case, the hard water concentration of the final solution is to be determined by titration and recorded.

Preparation of Test Tubes and Test Substance

- All test tubes that will receive test substance are thoroughly cleaned and steam sterilized prior to use.
- Test substance is prepared by dilution (1:1:10) by the addition of 1 part of Sterilex Ultra Disinfectant Cleaner: Solution 1 to 1 part of Sterilex Ultra Activator Solution to 10 parts AOAC Synthetic Hard Water.
- 10 ml of the prepared test substance is transferred by sterile disposable serological pipet, or other means as appropriate, into each 25 x 100 mm test tube designated for that purpose, and equilibrated to test temperature for ≥ 10 minutes prior to initiating testing or recording test substance temperature.

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- This substance is to be used within 3 hours of preparation.

Preparation of Test Tubes for Subculture/Neutralization and Incubation of Treated Carriers

- Before the test begins, the subculture/neutralization test tubes are prepared by cleaning, followed by the addition of 10 ml of an appropriate subculture neutralizing medium and steam sterilized prior to use.

Preparation of Test Carriers

- Before the test, clean stainless steel carriers are soaked in fresh 1M NaOH for at least 12 hours.
- Carriers are thoroughly rinsed using multiple tap-water rinses followed by a double R/O water rinse.
- An aliquot of rinse water from the final R/O water rinse is collected, and mixed with 2—3 drops phenolphthalein. If alkalinity is observed (rinse water turns pink) the carriers are re-rinsed until alkalinity is no longer observed.
- Carriers are distributed into an appropriate autoclavable container, covered with colonized or reverse osmosis water and steam sterilized.
- Carriers are allowed to cool to room temperature prior to use in the study.
- Prior to use in the study, carriers are observed for flaws and flawed carriers are discarded.

Preparation of Test Culture

- A daily culture of the test microorganism is created from the microbial library frozen stock culture in 10 ml AOAC Synthetic Broth or Nutrient Broth. This culture is incubated for 24 ± 2 hours at 36 ± 1°C.
- Subsequent daily transfers (<5) are made by transferring 0.010 ml of the most recent daily transfer culture into 10 ml AOAC Synthetic Broth or Nutrient Broth and incubated for 24 ± 2 hours at 36 ± 1°C. Only one daily transfer is required prior to initiation of the final test culture.
- A test culture is created by transferring 0.010 ml of the most recent daily transfer culture into an appropriate number of test tubes, each containing 10 ml AOAC Synthetic Broth or Nutrient Broth and incubated for 48-54 hours at 36±1°C.
- Test cultures are vortex mixed and allowed to stand at room temperature for ≥ 10 minutes.
- Remove the upper portion of the mixed culture(s), leaving behind any debris or clumps, and pool in an appropriate vessel(s).
- For the purpose of achieving carrier counts within the range of the study, dilution or concentration of the final test culture may be performed using the culture medium used to generate the test culture. Manipulation of the final test culture should be made prior to the addition of the organic soil load. Manipulated test cultures are used within 30 minutes for carrier inoculation.

Supplementation of Test Culture with Organic "Soil" Load

- Thawed, sterile fetal bovine serum is added to the pooled test culture such that the final concentration is 5.0 ± 0.5% (v/v) if applicable.
- The test culture-to-soil mixture is swirled gently to mix.

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Contamination of Carriers with Test Culture

- Deionized/reverse osmosis water is aspirated from the container containing the prepared carriers using a sterile serological pipet.
- The test culture is added to the drained vessel containing the pencylinders, such that all carriers are completely submerged in the test culture for uniform coverage (approximately 1 ml of culture per carrier).
- The test culture and pencylinders are allowed to stand for 15 ± 2 minutes at room temperature.
- After 15 ± 2 minutes have elapsed, the culture is aspirated and pencylinders are removed from the container aseptically using a sterile wire hook (carriers may be tapped or shaken prior to removal to remove excess culture) and are placed, no more than 12 carriers to a dish, on sterile double filter paper-lined, sterile Petri dishes. Carriers are placed on end, evenly spaced in the dish, such that they do not touch one another. If any carriers fall over, they are discarded from use in the test.
- Loaded Petri dishes are covered and allowed to dry. Final drying conditions will be recorded and detailed in the final report.
- Inoculated carriers are used within 2 hours of drying.

Exposure of Carriers to Test Substance

- Inoculated carriers are transferred, using a flame-sterilized wire hook, one carrier to each 25 x 100 mm test tube containing 10 ml test substance, at appropriate intervals to ensure careful and aseptic handling. Every attempt is made to ensure that carriers are not allowed to touch the sides of the test tube during this step. If a contaminated pencylinder touches the sides of the test tube going into the test substance, then the corresponding test tube is rejected.
- Tubes containing test substance and carrier are gently swirled then placed back in the water bath for the duration of the contact time.
- After the contact time for each carrier has elapsed, each carrier is removed from the test substance using a flame-sterilized wire hook. Carriers may be tapped in the lower third of the tube to remove excess test substance. Carriers are then transferred to a test tube containing 10 ml of the appropriate subculture/neutralization medium, such that it is completely submerged. If a treated pencylinder touches the sides of the test tube going into the test substance, then the corresponding test tube is rejected.
- Test tube racks are shaken and then incubated for 48 ± 2 hours at 36 ± 1°C.
- After incubation, the number of test tubes showing growth is recorded along with the number not showing growth.

Enumeration of Test Carriers

- Following the conclusion of the dry time, carriers are assayed in two sets of three; one set immediately prior to conducting the test, and one set immediately following the test. Each carrier is transferred individually to a subculture/neutralization test tube.
- These test tubes are placed in a beaker, filled with water to the level of liquid in the tubes, and held by hand in a sonicator so that the beaker bottom does not touch the bottom of the sonicator and all 3 liquid levels are approximately equal, and sonicated for 1 minute ± 5 seconds, timed with a certified digital timer.

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- After sonication, the test tubes are peeled for each set of three carriers, serially diluted in sterile PBS and plated in duplicate within 2 hours of sonication using standard dilution and plating techniques
- Enumeration plates are incubated for 48 ± 2 hours at 36 ± 1°C.
- The number of microorganisms present on the carriers after drying is determined using the following formula, including counts of "0," and excluding dilutions with counts of >300 * CFU = Colony Forming Units

$$\frac{(\text{Average CFU for } 10^{-1} + \text{Average CFU for } 10^{-2} + \text{Average CFU for } 10^{-3})}{10^3 + 10^4 + 10^5} = \text{CFU/ml}$$

$$(\text{CFU/ml}) \times 10^3 = \text{CFU/Carrier}$$

NOTE: Other dilutions may be plated in the event that a lower viable concentration is expected.

Neutralization Control

- A sterile uninoculated carrier is transferred to a test tube containing 10 ml of the test substance. After the specified contact time has elapsed, the carrier is transferred to a subculture/neutralization broth test tube, without allowing excess fluid to drain off of the carrier.
- After transfer, the test tube is inoculated with 10-100 CFU of test microorganism (obtained by serial dilution in PBS) and incubated along with the other test tubes.
- The inoculum is plated in duplicate to verify the number of CFU added and incubated alongside the test.

Viability Control

- One to two inoculated test carriers are placed in individual subculture/neutralization broth tubes and incubated alongside the test.

Subculture/Neutralization Sterility Control

- A test tube containing only subculture/neutralization broth is incubated alongside treated carriers.

Carrier Sterility Control

- A sterile uninoculated carrier is added to a test tube containing subculture/neutralization broth and is incubated alongside treated carriers.

Test Microorganism Purity Control

- A loopful of the test culture used in this study is subcultured to growth agar medium and incubated alongside enumeration plates to morphologically confirm presence of a pure culture.

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"Soil" Sterility Control

- An aliquot of soil is plated to sterile growth medium and incubated alongside enumeration plates to verify sterility at the time of test if applicable.

Media Sterility Control

- An aliquot of PBS is added to sterile growth medium and incubated alongside enumeration plates to verify sterility at the time of test.
- A plate containing only growth medium used in this study is incubated alongside enumeration plates to verify sterility at the time of test.

Incubation of Tubes and Enumeration and Control Plates

- All tubes and plates are incubated at $36 \pm 1^\circ\text{C}$ for 48 ± 2 hours.

Confirmation of Positive Tubes Following Incubation

- If multiple tubes demonstrate growth, $\geq 20\%$ of those tubes are confirmed not to be a result of contamination by plating on growth media, or other analysis as appropriate.
- All confirmatory plates are incubated for 18-24 hours at $36 \pm 1^\circ\text{C}$.

Confirmation of Negative Tubes Following Incubation

Note: Confirmation of negative tubes is conducted in the event that a specialized subculture neutralization media, such as Dey/Engley broth, is used which will not show turbidity.

- If all tubes demonstrate no growth, $\geq 20\%$ of those tubes are confirmed by plating on growth media, or other analysis as appropriate.
- All confirmatory plates are incubated for 18-24 hours at $36 \pm 1^\circ\text{C}$.

X. Success Criteria

- The experimental success (control) criteria follow:
 - The test microorganism must demonstrate a mean log density of at least 4.0 corresponding to a geometric mean density of 1×10^4 CFU/Corner.
 - The subculture/neutralization sterility control test tube is negative for growth.
 - The carrier sterility control test tube is negative for growth.
 - The viability growth control test tube(s) are positive for growth.
 - The neutralization control subculture/neutralization test tube is positive for growth.
 - The neutralization control inoculum demonstrates 10-100 CFU.

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- The "soil" sterility control is negative for growth if applicable
- The media sterility controls are negative for growth
- Restesting guidance for disinfection follows
 - When a test fails and the log₁₀ density of the test carriers is below 4.0, no retesting is necessary.

XII. Reporting

- Results are reported accurately and fully, in accordance with EPA GLP (40 CFR Part 160). A draft report will be provided for review by the Study Sponsor prior to study completion.

XIII. Data and Sample Retention

- The study report and corresponding data sheets will be held in the archives of Antimicrobial Test Laboratories for at least 2 years after the date of the final report and then may be destroyed. If the study is used by the Study Sponsor in support of a label claim, documentation may be returned to the Study Sponsor for archiving at Study Sponsor's expense
- The test substance may be returned to the Study Sponsor at Study Sponsor's request and expense within 30 days of study completion. If the Study Sponsor does not request return of the sample, it will be destroyed >30 days after study completion.

XIV. Quality Control

- The study is conducted in accordance with Antimicrobial Test Laboratories' Quality Management System and will undergo a full quality assurance review. All protocol amendments will be fully recorded and reported, as well as any deviations from the protocol.

XV. References

- Association of Official Analytical Chemists, International "AOAC Official Method 955.15 Testing Disinfectants Against Staphylococcus aureus. Revised 2013"
- U.S. EPA Product Performance Test Guidelines OCSPP B10.2200. Disinfectants for Use on Hard Surfaces - Efficacy Data Recommendations

XVI. Protocol Approval

"I, the Study Sponsor, have read and understand the study protocol. By signing this protocol I am certifying that the information and parameters accurately describe the test(s) to be completed in accordance with Good Laboratory Practice Standards (GLPS) stipulated by 40 CFR 160. I have also read, understood and agree to the terms and conditions listed in the protocol."

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Study Sponsor/Representative Signature Approving Protocol


Edward Fu, Sterilex, Sterilex Corporation

19 Jan 2018
Date


Nicholas Gacic, Study Director, Antimicrobial Test Laboratories, LLC

19 Jan 2018
Date

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Certificate of Analysis

This analysis was conducted in compliance with GLP-AOAC Use-Dilution Method
Laboratories, Inc. Study Number 5H10-00.

Name: Sterile 1,300 mg/g Disinfectant Clorox Solution I
Lab Number: P1331-24A
Date of Analysis: January 4, 2013

Test	Result
Chlorine Amount	9.74%
Hydrogen Peroxide	6.95%

[Signature]
Brian A. Rec
Study Director
CLL Laboratories, Inc.

✓ ✓ ✓ ✓
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AOAC International® Approved Method™ for Use-Dilution Test for Antimicrobial Agents

Certificate of Analysis

This analysis was performed in compliance with GLP-AOAC Test as part of Client
Characterization, Inc. Study Number 5810-01.

Sample: **Sample C, Blue Hydrogen Peroxide Standard I**
Lot Number: **A041-2548**
Date of Analysis: **March 4, 2014**

Test	Result
Chlorine	0.77%
Hydrogen Peroxide	0.63%

Brian A. Ross
Brian A. Ross
Study Director
Cure Laboratories, Inc.

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Units